Analysis of conformational stability of abnormal prion protein aggregates across the spectrum of Creutzfeldt-Jakob disease prions

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ABSTRACT

The wide phenotypic variability of prion diseases is thought to depend on the interaction of a host genotype with prion strains that have self-perpetuating biological properties enciphered in distinct conformations of the misfolded prion protein, PrP^{Sc}. The latter concept is largely based on indirect approaches studying the effect of proteases or denaturing agents on the physicochemical properties of PrP^{Sc} aggregates. Furthermore, most data come from studies on rodent-adapted prion strains, making current understanding of the molecular basis of strains and phenotypic variability in naturally occurring diseases, especially in humans, more limited. To fill this gap, we studied the effect of guanidine hydrochloride (GdnHCl) and heating on PrP^{Sc} aggregates extracted from 60 sporadic CJD and 6 variant CJD brains. While denaturation curves obtained after exposure of PrP^{Sc} to increasing GndHCl concentrations showed a similar profile among the 7 CJD types analysed, PrP^{Sc} exposure to increasing temperature revealed significantly different and type-specific responses. In particular, MM1 and VV2, the most prevalent and faster replicating CJD types, showed stable and highly resistant PrP^{Sc} aggregates, whereas VV1, a rare and slow propagating type, revealed unstable aggregates that easily dissolved at low temperature. Taken together our results indicate that the molecular interactions mediating the aggregation state of PrP^{Sc}, possibly enciphering strain diversity, are differently targeted by GdnHCl, temperature and proteases. Furthermore, the detected positive correlation between thermo-stability of PrP^{Sc} aggregates and disease transmission efficiency makes inconsistent the proposed hypothesis that a decrease in conformational stability of prions results in an increase in their replication efficiency.

IMPORTANCE

Prion strains are defined as infectious isolates propagating distinctive phenotypic traits after transmission to syngenic hosts. Although the molecular basis of prion strains is not fully understood, it is largely accepted that variations in prion protein conformation drive the molecular changes leading to the different phenotypes. In this study, we exposed abnormal prion protein aggregates, encompassing the spectrum of human prion strains, to both guanidine hydrochloride and thermal unfolding. Remarkably, while exposure to increasing temperature revealed significant strain-specific differences in the denaturation profile of the protein, treatment with guanidine hydrochloride did not. The findings suggest that thermal and chemical denaturation perturb the structure of prion protein aggregates differently. Moreover, since the most thermo-stable prion protein types were those associated with the most prevalent phenotypes and most rapidly and efficiently transmitting strains, the results suggest a direct correlation between the strain replication efficiency and the thermo-stability of prion protein aggregates.

INTRODUCTION

Prion diseases are invariably fatal neurodegenerative disorders of humans and other mammals, characterized by tissue deposition of aggregates of a misfolded, beta-sheet rich and partially protease-resistant isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}). In prion diseases misfolded PrP^{Sc}, originating exogenously or spontaneously, is thought to template the structural conversion of the host-encoded PrP^{C} in an autocatalytic process (1, 2). Intriguingly, a wealth of recent evidence indicates that proteinaceous seeds serving as self-propagating prion-like agents may represent a common pathogenetic mechanism in most, if not all, neurodegenerative diseases (3). Despite their relative rarity, prion diseases show a wide spectrum of clinical and pathological phenotypes with significant heterogeneity in disease duration, symptomatology and distribution or type of brain lesions. Current classification of sporadic Creutzfeldt-Jakob disease (sCJD), the most common human prion disease, includes six major disease phenotypes that strongly correlate at the molecular level with the genotype at the polymorphic codon 129 (methionine, M, or valine, V) of the *PRNP* gene (which encodes for PrP^{C}) and two PrP^{Sc} profiles or types (type 1 and type 2) comprising distinctive physicochemical properties such as size after protease treatment (respectively 21 and 19 kDa) and glycoform ratio (4, 5). Recent studies in animal models have shown that phenotypic variations among sCJD phenotypes are largely maintained after transmission into genetically defined hosts, suggesting that different prion strains are the main cause of this diversity (6–11). Although it is well established that PrP^{C} conversion into PrP^{Sc} involves consistent changes in the secondary structure with part of the α-helical structure turning into a β-sheet (12, 13), a complete structural characterization of PrP^{Sc} has been hampered by the propensity of the misfolded protein to form highly aggregated and detergent-insoluble polymers. Consequently, due to the limited data available from direct structural studies (14, 15), the putative central role of PrP^{Sc} tertiary or quaternary structure in determining the molecular basis of prion strains is not yet clearly demonstrated**.** Several experimental data, however,

indirectly support this hypothesis, both in yeast (16) and in mammals. For example, it is largely believed that the heterogeneity in fragment profile of proteinase K (PK)-digested PrP^{Sc}, which distinguishes at least some of the known prion strains, is the direct consequence of PrP^{Sc} aggregates having distinct conformations (17–21). Similarly, sedimentation profiles and protease sensitivity have been used as indirect markers of PrP^{Sc} structure, and have shown a correlation with strain-specific transmission properties (22–27). More recently, studies with rodent-adapted, cloned prion strains, demonstrated that also the conformational stability of PrP^{Sc}, measured indirectly either by inducing a progressive denaturation of the protein with the chaotropic salt guanidine hydrochloride (GdnHCl) or by exposing the protein to increasing temperatures in the presence of SDS (sodium dodecyl sulfate), may vary among different strains (28–30). Attempts have also been made to correlate PrP^{Sc} conformational stability to strain specific properties such as replication rates, although with conflicting, opposite results in murine and hamster models (28–30).

Overall, despite the wealth of experimental data collected in rodents and some evidence obtained in humans suggesting that phenotypic diversity is somehow related to distinct PrP^{Sc} isoforms with distinct structure, the conformational spectrum of these isoforms and their relationship to the issue of prions strains are still poorly understood. In particular, a systematic analysis of the conformational stability of PrP^{Sc} aggregates across the spectrum of human prions CJD is still lacking. Previous studies focused on the comparison between sCJD subtypes MM1 and MM2 (31–33) or between variant CJD (vCJD) and the most common sCJD type MM1 (34). Furthermore, only the unfolding induced by GdnHCl was addressed whereas the thermo-stability of PrP^{Sc} has never been explored in CJD.

To add insights to the intriguing relationship among PrP^{Sc} molecular types, the conformational stability of abnormal PrP^{Sc} aggregates, and the phenotypic expression of disease, we have

evaluated both guanidine-induced unfolding and thermo-stability of PrP^{Sc} across the whole spectrum of currently characterized human CJD strains.

MATERIALS AND METHODS.

Patients and tissues. We studied brain tissues from 60 cases of sCJD and 6 cases of vCJD. sCJD tissues included the whole spectrum of pure phenotypic variants recognized by current classification (5): 12 MM1, 9 VV1, 10 MV 2K, 12 VV2, 7 MM2-cortical (MM 2C), and 4 MM2-thalamic (MM 2T). In addition, 6 brains of sCJD MM 1+2C, the most common sCJD subtype with mixed histopathologic features and the co-occurrence of PrP^{Sc} types 1 and 2 were analyzed. Each sCJD brain was classified as a "pure" or "mixed" type based on the results of histopathological examination, PrP immunohistochemistry and PrP^{Sc} typing in multiple brain regions, according to Parchi et al (5). Unfixed brain tissues were obtained at autopsy and kept frozen at −80 °C until use. All samples used in this study were taken from the cerebral cortex of the frontal lobe.

Antibodies. The following monoclonal mouse antibodies, immunoreactive with human PrP, were used: 3F4 at 1:30000, which recognizes residues 106-110 (35), 12B2, at 1:8000, which binds residues 89-93 (36), and SAF60 at 1:2000, which reacts with residues 157-161 (37). In addition, the PrP^{Sc} type 2-specific polyclonal antibody T2 (1:5000), which binds residues 97-103 (7) and the rabbit antiserum 2301 (1:3000) to human PrP residues 220-231 were used.

Preparation of Total Brain Homogenates (THs). After removing any residual white matter from the cortical tissue sample, 50-100 mgs of gray matter were homogenized (20%, w/v) in TN-NP40 (100 mM Tris, 130 mM NaCl, 0,5% Nonidet P-40) at pH 7.4 (38) for the guanidine assay and (10%, w/v) in LB100 (100 mM Tris, 100 mM NaCl, 10 mM EDTA, 0,5% Nonidet P-40, 0,5% sodium deoxycholate) at pH 6.9 (39), for the thermo-solubilisation assay (TSA). In a subset of experiments having the specific purpose of reproducing a previously published protocol (32),

a clearing spin of THs at 3000 rpm for 10 minutes was performed. Total protein concentration was measured using a standard colorimetric method based on bicinchoninic acid (Pierce Biotechnology, Rockford, IL, USA).

Guanidine induced unfolding/refolding assays. Total brain homogenates (TH) were adjusted to a protein concentration of 5.5 mg/ml before denaturation. Equal volumes of TH and GdnHCl solutions ranging from 0 to 4 M (final concentration, Δ[GndHCl]=0.25M) were mixed and incubated for 1 h at 37°C at 300 rpm (Thermomixer Confort, Eppendorf). After the addition of PK at a final concentration of 8 U/ml, samples were re-incubated for another 1 h at 37°C at 300 rpm. Protease treatment was terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 3.6 mM. Samples were then precipitated in pre-chilled methanol for at least 3 hrs at -20° C, re-suspended in sample buffer (final concentration 3% SDS, 4% β-mercaptoethanol, 10% glycerol, 2 mm EDTA, 62.5 mm Tris, pH 6.8) and boiled for 6 minutes. Appropriate GdnHCl working concentrations were obtained from serial dilution of a 8M stock solution (Thermo Scientific Pierce, Protein Biology Products).

To monitor PrP^{Sc} refolding, after incubation with GdnHCl, samples were immediately diluted with 19 volumes of TN-NP40 and subsequently PK-digested at the same working conditions specified above, precipitated in methanol and re-suspended in sample buffer. To monitor reproducibility each treatment was repeated twice.

In a subset of samples from 6 MM1 and 6 MM 2C brains we also performed the conformational stability assay (CSA), after incubation with GdnHCl, according to a previously published protocol (32). Briefly, after a clearing spin at 3000 rpm for 10 minutes, aliquots of TH were mixed with aliquots of GdnHCl stock solution to have a final concentration of GdnHCl ranging from 0 to 4.0 M. After 1.5 hours of incubation at room temperature, samples were precipitated with 5-fold pre-chilled methanol, centrifuged at 16,000 *g* for 30 minutes at 4 ºC, and re-

suspended by sonication in 20 µl of LB100 (pH 8.0). Each aliquot was digested with 5 U/ml PK for 1 hour at 37 ºC. The reaction was stopped with 2 mM PMSF.

Thermo-solubilisation assays. TSA was performed according to Bett and colleagues with minor modifications (30). Briefly, TH were digested with 8 U/ml PK for 1h at 37°C with mild shaking (300 rpm). PK digestion was inactivated with PMSF (3,6 mM final concentration). Aliquots were mixed with an equal volume of loading buffer (final concentration 1,5% SDS, 2% β-mercaptoethanol, 5% glycerol, 1 mM EDTA, 31,25 mM Tris, pH 6.8) and heated to temperatures ranging from 25°C to 95°C (ΔT=10°C) for 6 min with shaking in a thermomixer at 1000 rpm before loading. To monitor reproducibility each treatment was repeated twice.

Western blot and quantitative analysis of protein signal. After boiling (or heating treatment) proteins were resolved in 13% polyacrylamide gels using a medium-sized gel electrophoresis apparatus (Criterion, Bio-Rad) and transferred to Immobilon-P membranes (Millipore Corp., Billerica, MA). After blocking in 10% nonfat milk in Tween-Tris-buffered saline, membranes were incubated with the primary antibody. After four washings in Tween-Tris-buffered saline, membranes were incubated for 1 h with an anti-mouse or an anti-rabbit secondary antibody conjugated to horseradish peroxidase (GE Healthcare; working dilution, 1:4000 or 1:3000) and washed again four times in Tween-Tris-buffered saline. The immunoreactive signal was detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore) on a LAS 3000 camera (Fujifilm Corp., Tokyo, Japan). Western blot signals were measured by densitometry using the software AIDA (Image Data Analyzer v.4.15, Raytest, Isotopenmessgeraete GmbH, Straubenhardt, Germany).

For guanidine unfolding assay curves were obtained by plotting the percentage of protein remaining after denaturation and PK-digestion treatments (with respect to the PK-digested but GndHCl-untreated sample i.e. 0 M, referred as $\%PrP^{fold}$) against the corresponding guanidine concentration.

ED_{50} ($[GndHCl]_{50}$, *e.g.* the guanidine concentration needed to unfold 50% of PrP^{Sc}) for each sample was calculated from the equation describing the sigmoidal curve that best fitted the densitometric data ($R^2 \geq 0.95$). To compare groups we also considered the percentage of protein remaining after denaturation plus PK digestion at [GndHCl] = 2 M (referred as $\%PrP^{fold}_{2M}$) and the percentage of protein detected after refolding plus PK digestion at [GndHCl] = 2M (referred as $\%PrP^{refold}_{2M}$). Refolded PrP^{Sc} was calculated by means of the formula (R-F)/N, where R = PrP^{Sc} signal detected after GdnHCl incubation, dilution and PK digestion, F = PrP^{Sc} signal detected after GdnHCl incubation without dilution and PK digestion, N = PrP^{Sc} signal detected after PK digestion without GdnHCl addition.

For the TSA we plotted the percentage of protein solubilized after heating treatment (with respect to the sample treated at 95°C, referred as $\%PrP^{Sc}_{mon}$) against the corresponding heating temperature. The ED_{50} (T_{50}, *e.g.* the temperature needed to solubilize 50% of PrP^{Sc}) for each sample was calculated from the equation describing the sigmoidal curve that best fitted the data ($R^2 \geq 0.95$). To compare groups we also considered the percentage of protein remaining after treatment at T = 35°C (referred as $\%PrP^{Sc}_{mon}35°C$) and at T = 75°C (referred as $\%PrP^{Sc}_{mon}75°C$).

Statistical analyses. Statistical analyses were performed with Sigma Plot 12.5 (Systat Software Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by all pairwise multiple comparison procedures were used to look for significant differences in the chosen parameters among CJD variants.

RESULTS

Analysis of molecular mechanisms associated with GndHCl induced unfolding of human PrP^{Sc}. It is well established that the exposure of PrP^{Sc} to increasing concentrations of GdnHCl progressively makes the protein more sensitive to protease digestion. Moreover, while a larger than 3 M concentration of the chaotropic denaturant irreversibly modifies PrP^{Sc} structure and protease-resistance, milder conditions allow the structural change to be reversible (38). Studies on PrP^{Sc} extracted from Syrian hamster brains also showed that GdnHCl induced unfolding mainly involves the N-terminal portion of PrP^{Sc}, as suggested by the persistence of a PK-resistant, GPI–linked, proteolytic fragment of about 16 kDa over a wide range of GdnHCl concentrations (40).

Before proceeding with the systematic analysis of the effects of GdnHCl-induced unfolding on human prions, we carried out preliminary studies aimed to select the most appropriate experimental conditions. We first compared the effect of PK digestion when performed before or after GdnHCl dilution, and unexpectedly found that the concentration of the denaturing agent that is required to fully digest PrP27-30 was significantly lower when PK was added to the undiluted sample. Indeed, after GdnHCl serial dilution we noticed a rapid partial recovery of PrP^{Sc} protease-resistance. Only treatments with GdnHCl concentrations of 3.5-4 M irreversibly denatured the protein after solubilisation and made it completely PK-sensitive (Fig. 1). Given the observed rapid recovery of PrP^{Sc} original conformation and the notion that PK is not significantly inhibited by GdnHCl even at a relatively high concentration (41, 42), we chose to perform PK digestions in the undiluted denaturing agent (i.e. PK hydrolysis in buffer containing GdnHCl). To select the most appropriate antibody for monitoring the effect of GdnHCl on PrP^{Sc} PK-sensitivity, we compared the 3F4 antibody, which recognizes a central PrP epitope (35), with antibodies against distinct C-terminal epitopes, which are known to recognize PrP^{Sc} truncated fragments, in addition to PrP^{Sc} 27-30 (20). In contrast to 3F4, which only revealed PrP^{Sc} 27-30,

C-terminal antibodies also showed variable amounts of a 18 kDa fragment, consistent with PrP^{C}-C1 (43), and of a 7-8 kDa fragment, likely generated by endogenous proteolysis of PrP^{C} during the one hour incubation at 37°C preceding PK-digestion. Given the presence of these fragments in untreated control samples and the lack of any correlation between the relative amount of such fragments and GdnHCl concentration, we concluded that: (i) the generation of these PrP^{Sc} fragments is not denaturation-dependent, (ii) central antibodies such as 3F4 are more appropriate than C-terminal antibodies to study the effect of GdnHCl on PrP^{Sc} structure.

PrP^{Sc} isolates associated with distinct human prion strains show similar denaturation curves after exposure to GndHCl. Guanidine denaturation curves showed a similar sigmoid profile in all CJD samples analysed (Fig. 2A, B). The curve that best fitted the data ($R^2 \geq 0.95$) was a four parameter sigmoid equation. Calculated values for $[GdnHCl]_{50}$ ranged from 0,86 M (sCJDVV2) to 1,03 M (sCJDMM 2T) with no statistically significant differences among CJD types (Fig. 2C). A similar value trend was obtained by calculating the percentage of detectable PrP^{Sc} signal at a GndHCl concentration of 2 M ($\%PrP^{fold}_{2M}$) (Fig. 2D).

To further look for strain-specific effects of GdnHCl, we also calculated the percentage of refolded PrP^{Sc} ($\%PrP^{refold}_{2M}$; see methods) at a given, intermediate GdnHCl concentration (2 M). In agreement with our preliminary screening, PrP^{Sc} renaturation occurred in all groups analysed. Calculated values ranged from 14% to 29%, but, again, with no statistically significant differences among CJD types (Table 1).

In order to exclude that the divergent results previously obtained by Cali et al. (32) on MM1 and MM 2C prions would only reflect a difference in the methodology used, we also repeated the experiments in a subgroup of samples following their protocol (32). The results obtained confirmed the lack of remarkable differences between MM1 and MM 2C (Fig. 3). In particular, the $[GdnHCl]_{50}$ values obtained in both groups (1.98 ± 0.05 M and 1.87 ± 0.09 M, respectively)

were intermediate between those previously observed in MM 2C (2.76 M) and MM1 (1.42 M) (32).

PrP^{Sc} aggregates associated with distinct human prion strains show a divergent response to thermal solubilisation. It has been shown that the exposure of PK digested PrP^{Sc} to a thermal gradient in the presence of SDS induces a progressive "solubilisation" of protein aggregates which can be measured by a semi-quantitative immunoblot analysis of monomeric PrP^{Sc} (30). By applying this experimental approach to the full spectrum of human prions, we found that, at variance with the GdnHCl assay, the specific profile of the calculated solubilisation curve and of T_{50}, $\%PrP^{Sc}_{mon}35°C$ and $\%PrP^{Sc}_{mon}75°C$ associated values, varied significantly according to the CJD type (Fig. 4 and 5). Overall, while PrP^{Sc} aggregates in sCJD VV1, and to a lesser extent in MM 2T or MM 2C showed a relatively high sensitivity to thermal solubilisation, those associated with MM1, VV2, and MV 2K were significantly more resistant. Hence, on the basis of the analysed parameters, CJD types could be grossly classified in three groups: resistant (MM1, VV2, MV2K), sensitive (vCJD, MM2C, and MM2T) and highly sensitive (VV1) to thermal solubilisation. A further heterogeneity was observed within the sensitive group with vCJD prions showing a more "resistant" profile at the highest temperatures in comparison to MM 2T and MM 2C (Fig. 5D).

To exclude the possibility that the observed heterogeneity in the thermo-stability of PrP^{Sc} aggregates derives from conformational changes that are limited to the 3F4 binding region, we re-analyzed a subgroup of MM1 and VV1 samples using the mAb SAF60. The thermo-solubilisation curves calculated from the immunoblots labeled with SAF60 fully matched those obtained using 3F4 (Fig. 4). In addition, the 13 kDa C-terminal fragment that is visualized by this antibody (20) in addition to PrP27-30, showed a solubilization kinetics that paralleled that of PrP27-30 in each CJD type (e.g. more thermostable in MM1 than in VV1) (Fig. 4). The latter

observation strongly suggests that PrP^{Sc} aggregates in CJD MM1 and VV1 include both fragments.

Finally we plotted the solubilisation curves for each of the three PrP^{Sc} glycoforms and found a similar thermo-solubilisation kinetics for each of them with only a minor trend toward a preferential solubilisation of the di- and monoglycosylated forms (data not shown).

PrP^{Sc} types co-occurrence in mixed sCJD phenotypes does not alter/affect thermal solubilisation properties of coexisting isoforms. It is well known that PrP^{Sc} types 1 and 2 coexist within the same brain in about 35% of sCJD cases (5). Accordingly, mixed phenotypes have been considered as distinct subtypes in current sCJD classification (44). However, the critical question of whether the brain co-occurrence of PrP^{Sc} types simply reflects the neutral coexistence of two prion strains forming independent protein aggregates, or in contrast represents interacting strains forming mixed aggregates with distinct physicochemical properties, remains unanswered. To investigate this issue we selected 6 cortical samples from sCJD MM1+2C containing a significant amount of both types (e.g. with the less represented type 2 being between 30 and 50% of the total PrP^{Sc} signal). We calculated the solubilisation curves for these samples using three different antibodies. More specifically, we monitored the solubilisation trend of the two PrP^{Sc} types simultaneously using 3F4, and analysed PrP^{Sc} types 1 and 2 separately, despite their co-occurrence, with the type-specific antibodies 12B2 and T2 (Fig. 6A). Remarkably, the curves generated with the 12B2 and T2 antibodies fully matched or paralleled very closely those of the corresponding pure phenotype (Fig. 6B). Accordingly, no significant statistical differences were observed in the calculated $T_{50,}$ $\%PrP^{Sc}_{mon}35°C$ and $\%PrP^{Sc}_{mon}75°C$ values between mixed and pure samples for both PrP^{Sc} types 1 and 2. Moreover, statistical analysis confirmed the differences between PrP^{Sc} types 1 and 2 when analysed in mixed samples using the type-specific antibodies (Fig. 6C, D, E).

298 **DISCUSSION**.

299 The analysis of GdnHCl-induced unfolding by either the conformation-dependent immunoassay
300 (CDI), which measures the extent of epitope exposure, or the conformational stability assay
301 (CSA), which, instead, monitors the progressive loss of PK-resistance after exposure to
302 increasing concentrations of GdnHCl, has been extensively applied to the study of PrP^{Sc}
303 molecules. CDI analysis of PrP^{Sc} unfolding in different murine prion strains yielded unique
304 binding profiles, suggesting that each strain is related to a specific PrP^{Sc} conformation (45).
305 Similarly, the stability of PrP^{Sc} aggregates measured by CSA or by a thermal stability assay
306 (TSA) was found strain-dependent and inversely correlated with the capacity to induce a rapidly
307 lethal disease in murine models (30). At variance with experimentally cloned rodent prion
308 strains, however, less numerous and less conclusive data have been obtained with PrP^{Sc}
309 preparations extracted from naturally occurring prion diseases, especially in humans. In the few
310 studies performed to date, sCJDMM1 PrP^{Sc} was shown to be more stable than MM 2C PrP^{Sc} by
311 both CDI and CSA, and MM1 PrP^{Sc} to have a moderately higher stability than vCJD PrP^{Sc} by
312 CDI (32–34). In the present study, we have carried out the first systematic analysis of PrP^{Sc}
313 conformational stability in a large series of brain samples across the whole spectrum of human
314 sCJD and vCJD prions. Our results failed to reveal significant strain-specific differences in the
315 GdnHCl-denaturation curve of PrP^{Sc} aggregates. Thus, despite the limitations related to the
316 methodology being rather crude, the data suggest that the intermolecular interactions modulating
317 the divergent PrP^{Sc} aggregation propensity among CJD types (27) are not strongly targeted by
318 GdnHCl.

319 As far as CSA is concerned, we introduced some variations in the protocol to minimize PrP^{Sc}
320 refolding, namely by performing the PK digestion step without removing or diluting GdnHCl.
321 Given that PrP^{Sc} refolding is paralleled by an increase in PK-resistance of the protein, it is
322 expected that GndHCl dilution before PK treatment would increase the calculated $[GndHCl]_{50}$,

and, indeed, $[GndHCl]_{50}$ values in previous studies were greater than 1.5 M, whereas our mean values ranged from 0.86 M to 1.03 M. In order to exclude the possibility that the discrepant results with a previous study (32) concerning MM1 and MM 2C prions, could be due to a difference in the methodology used, we repeated the study of GndHCl induced PrP^{Sc} unfolding using the original protocol (32). While, as expected, the changed procedure led to an increase in $[GndHCl]_{50}$ values, the results confirmed the lack of significant differences in the GdnHCl-denaturation curve between MM1 and MM 2C sCJD prions.

Another approach that has been used to characterize prion strains in mice (30, 46), although never applied to the study of CJD prions, focuses on the denaturing effect of heating in the presence of SDS. Likewise GndHCl, this assay likely mainly measures the propensity of PK-digested PrP^{Sc} aggregates to depolymerize. At variance with GdnHCl, however, the exposure to increasing temperatures revealed significantly different responses among sCJD types with MM1, VV2 and MV 2K PrP^{Sc} mainly forming stable, highly resistant aggregates, the VV1 type comprising highly unstable aggregates that easily dissolve at a relatively low temperature, and MM 2C, MM 2T and vCJD prions exhibiting an intermediate behavior.

The relatively high level of PrP^{Sc} that is solubilized at relatively low temperatures in some prion types is intriguing, and may suggest that these agents comprise different forms of PrP^{Sc}. Consistently, an increasing number of studies support the idea that a variable proportion of abnormal PrP is made by a soluble, poorly aggregated and fairly PK-sensitive form, although its specific role and relevance for disease pathogenesis remain controversial (27, 47). Our approach, focusing on PK-treated $PrP^{Sc,}$ did not allow us to address in depth the issue of the influence of this putative "fully PK-sensitive PrP^{Sc}" on thermostability profile; nevertheless the lack of an obvious correlation between sedimentation profile (as determined in 27), and thermostability (present work) of PK-treated PrP^{Sc} suggest that factors other than size also contribute to the thermo-stability of PrP^{Sc} aggregates in CJD.

The present results, when combined with the growing knowledge on human prion strains, provide novel insights into the relationships between the physicochemical properties of PrP^{Sc} aggregates and disease characteristics such as incidence, phenotypic expression, and transmission properties (5,7,10,11,48-51). In particular, PrP^{Sc} thermo-stability appears to largely, although not entirely, correlate with prion replication efficiency expressed by either attack rate and incubation time after experimental transmission in the most compatible host genotype or by relative incidence and duration of clinical disease (Table 2). In this regard, the comparison between VV2 and VV1 human prions appears to be particularly illustrative. Indeed, when compared to VV1, VV2 prions are more common, cause a more rapid disease (5), are more readily transmissible (10), and produce PrP^{Sc} aggregates with a higher thermo-stability, PK-resistance, and aggregation propensity (Table 2) (27). Thus, at variance with earlier observations in murine and yeast prions [PSI+], suggesting that a low PrP^{Sc} aggregate stability, by favoring the fragmentation of PrP^{Sc} aggregates, would result in a higher prion replication rate, in humans the thermo-stability of PrP^{Sc} seems to positively correlate with disease severity and "virulence" in the most compatible host genotype. It is noteworthy that our observation is also consistent with data obtained with hamster-adapted prion strains, showing that short incubation period strains are characterized by a higher conformational stability of PrP^{Sc} and a more efficient replication (28, 29).

Our results also reveal a relatively high thermo-stability, comparable to that of VV2, for MM1 and MV 2K prions. For the latter, the results are consistent with data from transmission studies showing that sCJD VV2 and MV 2K are linked to the same prion strain (V2) (9), whereas the result obtained with MM1 further confirms the positive correlation between PrP^{Sc} thermo-stability and strain "virulence" (Table 2). However, the degree of PK-resistance of PrP^{Sc} aggregates in MM1 is not as high as in VV2 and MV 2K (27), indicating that the two variables are not necessarily directly correlated (Table 2). Our findings that PrP^{Sc} aggregates in vCJD

show a very high resistance to PK digestion (27) despite being less thermo-stable than those from sCJD MM1 prions also support this conclusion. It has been suggested that PK itself may act as disaggregating agent by eliminating PrP monomers (48) and thus changing the equilibrium between monomers and polymers in favor of monomers. Likewise, GndHCl or heating exposure also act as disaggregating agent. Thus, the divergent response we obtained using these three methods suggest that propensity of PrP^{Sc} to disaggregate is significantly affected by the type of treatment, which is also in line with evidence indicating that temperature and GdnHCl destabilize the folded structure of proteins by means of distinct molecular mechanisms (52–55).

In addition to the analyses on pure phenotypes, we performed TSA on six cases with a mixed phenotype, carrying MM at *PRNP* codon 129, and showing the co-occurrence of PrP^{Sc} types 1 and 2 in the brain. In particular, we addressed the unsolved question of whether the brain co-occurrence of PrP^{Sc} types simply reflects the neutral coexistence of two prion strains forming independent protein aggregates or, in contrast, interacting strains form mixed aggregates with novel specific properties. At variance with the findings of a previous study (32) arguing that the co-existence of types 1 and 2 in the same anatomical region may confer special conformational characteristics to the mixed PrP^{Sc} type aggregate, the results we obtained using type-specific antibodies support the former hypothesis. Indeed, properties of each individual type, when co-existing, exactly fit those of the corresponding pure type, suggesting a lack of interaction between the two PrP^{Sc} types. The discrepant results between the two studies are difficult to explain given that they were obtained using different approaches (CSA vs TSA). Unfortunately, we could not address the issue further, since we failed to reveal a significant difference in PrP^{Sc} conformational stability between the pure MM1 and MM 2C types using CSA.

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397

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FIGURE LEGENDS

FIGURE 1. Monitoring PrP^{Sc} refolding after GdnHCl-induced unfolding. Samples were exposed to increasing concentrations of GdnHCl and then PK-digested immediately (upper panel) or after GdnHCl dilution (lower panel). PrP^{Sc} refolding is detected after GdnHCl dilution in samples exposed up to 2.5M GdnHCl.

FIGURE 2. GdnHCl-induced unfolding demonstrate a similar conformational stability of PrP^{Sc} aggregates across the spectrum of CJD prions. Brain homogenates were treated with increasing concentrations of GdnHCl and digested with PK, followed by SDS-PAGE and immunoblotting. Membranes were incubated with the primary antibody 3F4. (**A**) Representative immunoblots showing similar denaturation profiles among the seven CJD types analyzed. (**B**) Plots of GndHCl-induced PrP^{Sc} unfolding for each sCJD type and vCJD. The y axis reports the percentage of folded PrP^{Sc} (e.g. percentage of PK-resistant PrP^{Sc}) relative to the untreated sample. Symbols represent data expressed as mean ± standard deviation, lines the sigmoid curves that best fit the data. (**C**) $[GdnHCl]_{50}$ values for each CJD type, expressed as mean ± standard deviation, indicating the GdnHCl molar concentration necessary to unfold 50% of untreated PrP^{Sc}. (**D**) Percentage of folded PrP^{Sc} at 2 M GndHCl, expressed as mean ± standard deviation.

FIGURE 3. Conformational stability assay shows only subtle differences between sCJDMM1 and MM 2C prions. (**A**) Representative immunoblots of CSA, performed according to the protocol described by Cali et al. (32) on MM1 and MM 2C prions. Membranes were incubated with the primary antibody 3F4. (**B**) Plots of GndHCl-induced PrP^{Sc} unfolding data sets for MM1 and MM 2C prions. The y axis reports the percentage of folded PrP^{Sc} (e.g. percentage of PK-resistant PrP^{Sc}) relative to the untreated sample. Symbols represent data expressed as

mean ± standard deviation, lines the sigmoid curves (s) that best fit the data. $[GdnHCl]_{50}$ is expressed as mean ± standard deviation.

FIGURE 4. CJD types show remarkable differences in the thermal stability of PK-digested PrP^{Sc} aggregates. PK-digested brain samples were subjected to increasing temperatures, followed by SDS-PAGE and immunoblotting**.** Representative immunoblots for each CJD type, probed with the primary antibodies 3F4 (all types) and SAF60 (only for sCJDMM1 and VV1, as labeled) are shown.

FIGURE 5. Comparative analysis of the divergent thermal stability of CJD prions. (**A**) Plots of TSA data sets for each sCJD type and vCJD. The y axis reports the percentage of PrP^{Sc} in the monomeric state at each tested temperature relative to the sample treated at 95 °C. Symbols represent the data expressed as mean ± standard deviation, lines the sigmoid curves (s) that best fit the data. (**B**) T_{50} values for each tested CJD group, expressed as mean ± standard deviation, indicating the temperature necessary to unfold 50% of PrP^{Sc} relative to the sample treated at 95°C. N.E. = not estimable. (**C**) Percentage of monomeric PrP^{Sc} at 35 °C expressed as mean ± standard deviation. (**D**) Percentage of monomeric PrP^{Sc} at 75 °C expressed as mean ± standard deviation. In panels (**B-D**) the triple asterisk (***) indicates a $p<0.001$ for all pairwise multiple comparisons between the groups; the double asterisk (**) indicates a $p<0.001$ for all pairwise multiple comparisons with the exception of: (**B**) vCJD vs MM 2C ($p<0.005$) and vCJD vs MM 2T ($p=0.184$, not significant); (**C**) VV2 vs MM 2T ($p<0.002$), and VV1 vs vCJD ($p<0.005$); (**D**) MM 2C vs vCJD ($p<0.005$), VV1 vs vCJD ($p=0.016$), and MM 2T vs vCJD ($p=0.029$).

FIGURE 6. In vivo co-occurring MM1 and MM 2C prions maintain the distinctive thermo-stability of the corresponding pure CJD type. PK-digested brain samples were subjected to increasing temperatures, followed by SDS-PAGE and immunoblotting. (**A**) Representative immunoblots of TSA performed on a MM1+2C sample, probed with primary antibodies 3F4, 12B2 and T2. (**B**) Plots of temperature solubility assay data sets for PrP^{Sc} types 1 and 2, when co-occurring (MM1+2C) are compared to those of the corresponding pure type (MM1 and MM 2C respectively). Type-specific antibodies 12B2 and T2 have been used to obtain accurate separate measurement of type 1 and type 2 signals in mixed samples. Symbols represent data expressed as mean ± standard deviation, lines the sigmoid curves that best fit the data. (**C**) Comparison of T_{50} values between pure and mixed variants. The triple asterisk (***) indicates a statistically significant differences between groups at $p<0.001$. (**D**) Comparison of the percentage of monomeric PrP^{Sc} at 35 °C between pure and mixed variants. The double asterisk (**) indicates a $p<0.05$ between groups with the exception of MM1 vs type 2 PrP^{Sc} (not significant). (**E**) Comparison of the percentage of monomeric PrP^{Sc} at 75 °C between pure and mixed variants. The triple asterisk (***) indicates a $p<0.001$ between groups.

636 **TABLE 1.** Mean values of %PrP^{Sc}_{refold}2M for each CJD type[a]

CJD type	%PrP^{Sc}_{refold}2M
MM1	24.06 ± 11.69
VV2	21.32 ± 6.02
MV 2K	19.72 ± 15.53
MM 2C	24.53 ± 9.69
MM 2T	21.07 ± 10.73
VV1	11.19 ± 5.18
vCJD	17.60 ± 10.25

637 [a]All parameters are expressed as mean ± standard deviation; no statistically significant

638 differences were observed among CJD types.

TABLE 2. CJD strains: Comparison of epidemiological and clinical data, results of experimental transmission, and biochemical PrP^{Sc} properties.

CJD type	Strain	Incidence as % of total sCJD cases (ref.)	Mean disease duration in months (ref.)	Attack rate in % (ref.)[a,b]	Incubation time in days (ref.)[a,b]	T_{50} (°C) (present work)	PK resistance ED_{50} (U/ml)[c] (from ref. 27)
VV2	V2	15 (5)	6.3[d] (5)	100 (7, 10, 48)	274 ± 4 (10) 302 ± 9 (7, 48)	82.05 ± 3.70	4.137 ± 3.562
MV 2K		8 (5)		100 (10, 48)	288 ± 3 (10) 329 ± 3 (48)	79.48 ± 3.63	1.407 ± 0.892
MM1	M1	40 (5)	4.0 (5)	100 (7, 10, 48)	446 ± 3 (10) 467 ± 24 (7, 48)	79.66 ± 2.32	0.093 ± 0.068
vCJD	BSE	rare (49)	14 (49)	83 (50) 100 (50)	540 ± 41 (50) 668 ± 22 (50)	65.26 ± 3.19	5.192 ± 2.378
MM 2T	M 2T	1 (5)	15.5 (5)	93 (11)	535 ± 32 (11)	59.41 ± 6.04	0.134 ± 0.089
VV1	V1	1 (5)	15.3 (5)	50 (10)	568 ± 0 (10)	< 25	0.034 ± 0.028
MM 2C	M 2C	1 (5)	20.0 (5)	0 (10, 11, 48, 51)	- (10, 11, 48, 51)	57.11 ± 5.96	0.276 ± 0.210

[a] Data are from PrP-humanized knock-in mice expressing normal levels of human PrP^{C}.

[b] Data for each strain refer to those obtained in recipient animals carrying the most compatible *PRNP* 129 genotype.

[c] ED_{50} is the PK concentration needed to digest 50% of PrP^{Sc}.

[d] The reported mean disease duration only refers to VV2, the group with the most compatible 129 genotype to the V2 strain.

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